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Plasma Native and Peptidase-Derivable Met-Enkephalin Responses to Restraint Stress in Rats. Adaptation to Repeated Restraint

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Research Article

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Plasma Native and Peptidase-derivable Met-Enkephalin Responses to Restraint Stress in Rats

Adaptation to Repeated Restraint

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Abstract

Met-enkephalin and related proenkephalin A-derived peptides circulate in plasma at picomolar concentration as free, native pentapeptide and at nanomolar concentration in cryptic forms. We have optimized conditions for measurement of immunoreactive Met-enkephalin in plasma and for generation by trypsin and carboxypeptidase B of much greater amounts of total peptidase-derivable Met-enkephalin in plasma of rats, dogs, and humans. Free Met-enkephalin (11 pM) is constituted by native pentapeptide and its sulfoxide. Characterization of plasma total Met-enkephalin derived by peptidic hydrolysis revealed a small amount (38 pM) of Met-enkephalin associated with peptides of molecular mass < 30,000 D, and probably derived from proenkephalin A, but much larger amounts of Met-enkephalin associated with albumin (1.2 nM) and with a globulin-sized protein (2.8 nM). Thus, plasma protein precursors for peptidase-derivable Met-enkephalin differ structurally and chemically from proenkephalin A. Met-enkephalin generated from plasma by peptidic hydrolysis showed naloxone-reversible bioactivity comparable to synthetic Met-enkephalin.

Prolonged exposure of adult, male rats to restraint stress produced biphasic plasma responses, with peaks occurring at 30 s and 30 min in both free native and total peptidase-derivable Met-enkephalin. Repeated daily exposure to this 30-min stress resulted in adaptive loss of responses of both forms to acute restraint. Initial plasma responses of Met-enkephalin paralleled those of epinephrine and norepinephrine, but subsequently showed divergence of response. In conclusion, Met-enkephalin circulates in several forms, some of which may be derived from proteins other than proenkephalin A, and plasma levels of both free native, and peptidase-derivable Met-enkephalin are modulated physiologically. (*J. Clin. Invest.* 1990. 85:861–873.) proenkephalin A peptides • plasma opioid peptides • plasma neuropeptides • plasma sympathoadrenal

Preliminary reports of this work have been published in abstract form (1986, 1987. *Soc. Neurosci.* 12:410; 13:1303) and have been presented at the European Society for Neurochemistry, Prague, 1986, the Sixth International Catecholamine Symposium, Jerusalem, 1987, the Fourth Symposium on Catecholamines and Other Neurotransmitters in Stress, Smolenice, 1987, and the International Narcotic Research Conference, Albi, 1988.

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Introduction

Met-Enkephalin and a number of proenkephalin A-derived peptides are widely distributed within the central nervous system and in many peripheral tissues including adrenal medulla, sympathetic nerve terminals and ganglia, pancreas, gastrointestinal tract, and pituitary (1, 2). Co-secretion of enkephalin-related peptides and catecholamines has been demonstrated from chromaffin cells in vitro, from isolated perfused adrenal glands and from adrenal gland in vivo (3–7). Although circulating Met-enkephalin has been quantitated by a number of laboratories, considerable variability in plasma concentration has been described (8). Met-enkephalin is degraded rapidly in plasma and changes in circulating levels in response to stimuli have been difficult to document. Indeed, very little is known regarding the in vivo regulation of secretion of Met-enkephalin-related peptides and of the plasma levels of Met-enkephalin. It has been shown clearly that in a number of tissues, including adrenal medulla and brain, enkephalin-containing peptides are converted to Met-enkephalin in a series of enzymic steps which involve processing by trypsinlike and carboxypeptidase B-like enzymes (9–11). The Met-enkephalin in such peptides derived from proenkephalin A has been referred to as cryptic Met-enkephalin. It remains unclear whether Met-enkephalin is secreted into the circulation only as a pentapeptide or whether it may be secreted also in a larger protected form, and later hydrolyzed to the pentapeptide. In addition and analogous to other hormones, it seems probable that Met-enkephalin also circulates in a bound form, in association with albumin or a globulin.

The physiological relevance of circulating Met-enkephalin remains poorly understood. Inferences have been made from studies employing the opioid antagonist, naloxone. However, conclusions regarding effects of circulating Met-enkephalin, which can be drawn from such studies, have been necessarily limited since naloxone acts at receptors in both periphery and brain and since other circulating endogenous opioids such as β-endorphin might mediate such naloxone-antagonizable effects. Clearly, in order to better document the physiology of Met-enkephalin acting as a hormone, studies of its circulating forms and their regulation are essential. Thus, during the past few years we have determined to measure immunoreactive Met-enkephalin in plasma, to define more optimal enzymic conditions for the generation of peptidase-derivable Met-enkephalin in plasma, to characterize the large circulating forms of peptidase-derivable Met-enkephalin, and to define in rats the physiologic regulation of plasma responses to a psychological stress of free native Met-enkephalin and total peptidase-derivable Met-enkephalin.

Methods

Materials. Met-*O*-enkephalin sulfoxide, L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)[1]-treated trypsin, carboxypeptidase B,

1. *Abbreviations used in this paper:* TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.

trypsin inhibitor, rabbit γ-globulin, DL-dithiothreitol (DTT), EGTA, Sephadex G-200 fine, Tris-HCl, sodium azide, bovine serum albumin, propylene glycol, and chloral hydrate were obtained from Sigma Chemical Co., St. Louis, MO. Met-enkephalin, Leu-enkephalin, Tyr-Gly-Gly-Phe, Gly-Gly-Phe-Met, Met-enkephalin-Arg^6-Phe^7, Met-enkephalin-Arg^6-Gly^7-Leu^8, metorphamide, peptide B, peptide BAM-12P, peptide E, and peptide F were obtained from Peninsula Laboratories, Inc., Belmont, CA. Porapak Q (80–120 mesh) was obtained from Waters Associates-Millipore, Milford, MA; aprotinin (Trasylol) from Mobay, New York, and polyethyleneglycol (PEG), molecular mass 8,000 D, from Aldrich Chemical Co., Milwaukee, WI. Commercial antiserum, anti-Met-enkephalin 18R2, was obtained from Immunonuclear Corp., Stillwater, MN, and ^{125}I-Met-enkephalin was obtained from New England Nuclear-Dupont, Wilmington, DE. Disodium EDTA, citric acid, sodium phosphate monobasic and dibasic, and magnesium sulfate were obtained from Fisher Scientific Co., Fair Lawn, NJ; Spectrapor membrane tubing from Spectrum Medical Industries, Los Angeles, CA; Affi-Gel Blue (50–100 mesh wet) from Bio-Rad Laboratories, Richmond, CA; and Diaflo Ultrafilters PM30 and Centricon-100 from Amicon Corp., Danvers, MA. Eqithesin was made from 2.13 g of chloral hydrate, 1.04 g magnesium sulfate, 14.1 ml of propylene glycol, 9.75 ml of Nembutal, 3.78 ml of ethanol, and adjusted to 50 ml in volume with 22.4 ml of water.

Collection of blood. Blood for Met-enkephalin estimation was collected on ice in polypropylene tubes in EDTA 2.7 nmol/ml of blood, citric acid 17.7 μmol/ml of blood, and aprotinin 200 kIU/ml blood (12). Blood was centrifuged at 40,000 *g* for 30 min at 4°C, then plasma was acidified with 0.5 N HCl final concentration, and stored at −70°C until assay. Blood for norepinephrine and epinephrine to be measured by a single-isotope radioenzymatic assay (13) was collected into chilled polypropylene tubes containing 2 mg of DTT and 10 μl of 10% EGTA. Blood was centrifuged at 30,000 *g* for 10 min and the deproteinized plasma was stored at −70°C until assay.

Extraction of native Met-enkephalin from plasma. Acidified plasma, 300 μl, was neutralized with 2 ml of 0.06 M phosphate buffer, pH 10.2; this phosphate buffer was prepared from sodium phosphate monobasic 0.07 M and sodium phosphate dibasic 0.05 M, then pH adjusted with sodium hydroxide 10 N. Samples were applied to Porapak Q columns composed of 250 mg of Porapak Q in 3 ml of absolute ethanol (200 proof). Porapak Q slurry was prepared by degassing overnight 25 g in 350 ml of absolute ethanol. Shortly before applying the samples, columns were equilibrated with 6 ml of doubly distilled water; then loaded columns were washed with 3 ml of doubly distilled water, and Met-enkephalin was eluted with 3 ml of absolute ethanol, lyophilized overnight and assayed immediately.

Radioimmunoassay of Met-enkephalin in plasma. Commercial Met-enkephalin antiserum 18R2 raised in rabbit was examined for cross-reactivity with the following peptides: Met-*O*-enkephalin, Met-enkephalin, Tyr-Gly-Gly-Phe, Gly-Gly-Phe-Met, Leu-enkephalin, Met-enkephalin-Arg^6-Phe^7, Met-enkephalin-Arg^6-Gly^7-Leu^8, metorphamide, peptide B, peptide BAM-12P, peptide E, and peptide F.

Lyophilized samples were reconstituted with 100 μl of 0.06 M phosphate buffer, pH 6.5, containing 0.2% of bovine serum albumin and 0.002% sodium azide. 50 μl of antiserum diluted 1:10,000 and 50 μl of ^{125}I-Met-enkephalin (~ 1,500 cpm) were added and samples were incubated at 4°C. After 24 h of incubation, 50 μl of 1% rabbit γ-globulin were added and incubation was maintained at 4°C for 30 min. Separation of bound from free complex was performed by adding 250 μl of 25% PEG. After 30 min of incubation, samples were centrifuged at 2000 *g* at 4°C for 30 min, supernatant was discarded, and pellets were counted in γ-counter (Packard Instrument Co., Inc., Downers Grove, IL).

Recovery of Met-enkephalin from Porapak Q was studied by extraction of Met-enkephalin from phosphate buffer or plasma and comparison with unextracted Met-enkephalin standard. Recovery of immunoreactive Met-enkephalin standard from 0.5 N HCl was examined after lyophilization at pH 6.0, reconstitution in buffer and extraction with Porapak Q columns. Recovery of Met-enkephalin was also studied by comparing the recovery of ^{125}I-Met-enkephalin (~ 5 pg) from phosphate buffer or plasma. Parallelism of native endogenous Met-enkephalin with standard Met-enkephalin was demonstrated by comparison with assay of 100–1,000 μl of each of rat, human, and dog plasma.

Further validation of the authenticity of this immunoreactive Met-enkephalin was obtained by partial purification using reverse-phase HPLC on a C_{18} column 0.39 × 30 cm preceded by a guard column 4.6 × 30 mm (both columns, Waters Associates) with two systems: 20% acetonitrile in 0.1% trifluoroacetic acid, or 0.01 M phosphate buffer/methanol in a 70/30 ratio, at flow rates of 1–1.8 ml/min; 1-min fractions were collected for RIA of Met-enkephalin. HPLC was carried out in this laboratory by Dr. A. A. Houdi.

Total peptidase-derivable Met-enkephalin in plasma. To release Met-enkephalin from larger molecular forms present in plasma or from Met-enkephalin bound to plasma protein (together with free these comprise total Met-enkephalin), different conditions for enzymic hydrolysis were compared. Plasma was incubated for 30 min at 37°C with TPCK-treated trypsin in concentrations of 300–2,000 μg/ml. Immediately after, plasma was incubated for 15 min with carboxypeptidase B, 10–100 μg/ml plus trypsin inhibitor 2.5 mg/ml. From different combinations of trypsin and carboxypeptidase B described above, the optimal conditions were determined (Table I). In subsequent studies for preparation of total plasma Met-enkephalin, 50 μl of acidified plasma was treated with 75 μl of Tris-base buffer 0.5 M, pH 9.6, to bring pH to 7.8–8.0, then incubated with 50 μl of TPCK-treated trypsin (1 mg/ml Tris-HCl, pH 7.7) in water bath at 37°C for 30 min, followed by further incubation for 15 min with 50 μl of carboxypeptidase B (50 μg/ml Tris-HCl) plus trypsin inhibitor 2.5 mg/ml. Enzymic hydrolysis is stopped by addition of 250 μl of ice-cold Tris-HCl (pH 7.7) and placing samples on ice. Before applying to Porapak Q columns, 500 μl of 0.006 M phosphate buffer, pH 6.0, is added to change pH to 6.5, and samples are processed as described above for native Met-enkephalin.

Parallelism of total endogenous Met-enkephalin released after enzymic hydrolysis with Met-enkephalin standard was demonstrated by comparison with assay of 10–500 μl of each of rat, human, and dog plasma treated as described above with trypsin and carboxypeptidase B. This peptidase-derivable (total) Met-enkephalin is extracted on Porapak Q before radioimmunoassay.

Characterization of total peptidase-derivable Met-enkephalin in plasma. Circulating total peptidase-derivable Met-enkephalin may be derived from larger precursor peptides of molecular mass < 30,000 D, which are in turn derived from preproenkephalin A, or may be derived from other proteins unrelated to proenkephalin A but containing the Met-enkephalin sequences, or may be bound to plasma protein carriers. To determine the approximate size of the peptides containing or binding Met-enkephalin, plasma was filtered on a Diaflo Ultrafilters PM30 membrane. The filtrate (cutoff < 30,000 D) obtained by washing the membrane with saline was collected separately and subjected to enzymic hydrolysis with trypsin and carboxypeptidase B before measurement of Met-enkephalin by RIA. Subsequently, a portion of the eluate from Amicon membranes was mixed with Affi-Gel Blue in order to separate Met-enkephalin-associated albumin from Met-en-

Table I. Generation of Immunoreactive Met-enkephalin on Incubation of Plasma (1 ml) with Varying Concentrations of Trypsin and Carboxypeptidase B

	Trypsin			
			μg/ml	
Carboxypeptidase B	300	500	1,000	2,000
μg/ml			*pM*	
10	350	600	1,550	1,550
20	200	950	2,250	2,000
50	—	—	5,000	—
100	—	—	4,450	—

kephalin associated with other proteins. 2 ml of saline eluate from Amicon membranes containing 800 μl of plasma equivalents was incubated for 2 h with 5 ml Affi-Gel Blue; the slurry was centrifuged for 2 min at 900 *g* and the supernatant I was collected. The pellet was washed four times in 3 ml of sodium phosphate buffer 0.02 M, pH 7.1, centrifuged, and resuspended between each wash and the washes combined. The washed pellet was resuspended in 3 ml of sodium chloride 1.4 M in phosphate buffer, incubated for 2 h at 4°C, and then centrifuged, and the supernatant II was collected. Supernatant I, washings, and supernatant II were lyophilized and hydrolyzed separately with trypsin and carboxypeptidase B before measurement of Met-enkephalin by RIA. In a subsequent experiment, we compared the amount of peptidase-derivable Met-enkephalin released from filtrate and retentate, after filtration through a Centricon-100 (Amicon Corp.) membrane, of plasma which had been pretreated with guanidine 6 M plus mercaptoethanol 1 M vs. untreated plasma.

1 ml of rat plasma was dialyzed overnight through a Spectrapor membrane tubing (cutoff 10,000–12,000 D) against 2 liters of sodium phosphate buffer 0.02 M, pH 7.1. Separation of Met-enkephalin associated with protein or peptide > 12,000 D was carried out by size-exclusion chromatography; 250 μl of this dialysate was applied to Sephadex G-200 fine column 1.5 × 80 cm. Fractions of 2 ml of sodium phosphate buffer 0.02 M (0.2 ml/min) were collected. Aliquots taken for spectrophotometric assay of protein (Lowry) showed presence of two major protein peaks. Fractions were lyophilized and hydrolyzed with trypsin and carboxypeptidase B before measurement of Met-enkephalin by RIA. The chromatography on Sephadex G-200 fine column was repeated three times.

Bioactivity of peptidase-derivable Met-enkephalin. 60 ml of human plasma were digested with trypsin and carboxypeptidase B in our usual manner as described above to release total Met-enkephalin. After extraction on Porapak Q column and lyophilization of the eluate, the total Met-enkephalin was run on reverse-phase HPLC as described above. Fractions corresponding to Met-enkephalin and to Met-enkephalin sulfoxide were lyophilized individually, then dissolved in Krebs-Henseleit buffer for assay of bioactivity on mouse vas deferens (14). The ability of standard Met-enkephalin and Met-enkephalin obtained by peptidic hydrolysis of plasma to inhibit electrically induced contraction of strips of mouse vas deferens were compared in an assay kindly carried out with Drs. R. Altiere, A. Houdi, and J. Kiritsy-Roy (University of Kentucky). Aliquots representing three different plasma volumes covering a sevenfold range were compared in duplicate with four concentrations of Met-enkephalin standard.

Animal studies. Adult male Sprague-Dawley rats (Harlan-Sprague-Dawley, Inc., Indianapolis, IN) weighing 300–315 *g* were housed individually in an environmental room at 24°C with controlled light-dark cycles (lights from 0700 to 1900 h) and provided free access to food and water. 3 d before experimentation, rats received under Equithesin anesthesia an indwelling left carotid artery cannula which allowed sampling of blood and injection of drugs at intervals in conscious, freely moving animals without stress (15). A series of studies was carried out in which we examined: (*a*) the time courses of the plasma native and peptidase-derivable Met-enkephalin and catecholamine responses to brief and prolonged restraint stress; (*b*) the effect of frequent blood sampling on the levels of these parameters; (*c*) the effect of repeated restraint on the plasma Met-enkephalin and catecholamine responses to acute restraint stress. In all experiments, groups comprised 7–11 animals. In order to establish the time course of plasma Met-enkephalin responses to restraint stress, rats were stressed by placing them in plexiglass restraining holders (16) for 2 (one experiment), 10 (five experiments), or 30 min (five experiments). Blood was taken from control and experimental animals at 1 min before stress and at a number of intervals from 0.5 to 60 min after the onset of stress. Data from 11 experiments were combined to provide complete time courses. The maximum blood volume withdrawn at any time point was 0.8 ml and the maximum blood volume withdrawn from one rat in any experiment was 3.5 ml. The effect of frequent blood sampling on the levels of Met-enkephalin was examined using several protocols in these experiments. In two experiments, 260 μl of blood was withdrawn from control and stressed rats at 1 min before stress and at 0.5, 1, 1.5, 2, 2.5, 3, 5, 7, and 10 min after the onset of stress. In other experiments, 0.5 ml was withdrawn at 1 min before stress and at 2, 10, 20, and 30 min after the onset of stress, or 0.8 ml was withdrawn at 1 min before stress and at 1, 15, and 30 min after the onset of stress.

In order to determine whether repeated application of stress alters the plasma Met-enkephalin responses to stress, rats were stressed 30 min daily for 7 d by restraining in plastic holders. Blood was collected on days 1 and 7 at 1 min before and 1, 15, and 30 min after the onset of stress.

Data analysis. Data were analyzed by analysis of variance and Duncan's multiple range test.

Results

Characterization of RIA for Met-enkephalin in plasma. Recovery of Met-enkephalin standard over range of 0.4–200 pg in the RIA including extraction from Porapak Q was 80% from buffer and 95% from 1 ml of acidified plasma (after subtraction of the endogenous Met-enkephalin in this plasma). When standard Met-enkephalin was prepared in 0.5 N HCl (to mimic acidification conditions for handling the plasma), then brought to pH 6 with buffer, extracted on Porapak Q, and assayed, recovery was 75%. Recovery of ^{125}I-Met-enkephalin (20,000 cpm, ~ 5 pg) from Porapak Q was 80.0±3.1%; these data suggest that recovery of Met-enkephalin is absolute from aspects of the assay procedure other than extraction on Porapak Q (Fig. 1). Intra- and interassay coefficients of variation are 7% and 11%, respectively.

Cross-reactivity of a number of related peptides with antiserum to Met-enkephalin was studied (Fig. 2). Using 1 pmol of peptide, cross-reactivities were: Met-*O*-enkephalin sulfoxide, 160%; peptide F, 22%; peptide E, 2%; Leu-enkephalin, 1.8%; peptide B, 0.8%; Met-enkephalin-Arg^6-Phe^7, 0.6%; Met-enkephalin-Arg^6-Gly^7-Leu^8, 0.1%; metorphamide, 0.09%; peptide BAM 12P, 0.07%; Tyr-Gly-Gly-Phe and Gly-Gly-Phe-Met, < 0.01%. Thus, only Met-enkephalin sulfoxide and peptide F showed significant cross-reactivity with this Met-enkephalin antiserum.

Parallelism of native endogenous Met-enkephalin in plasma of rat, human, and dog with Met-enkephalin standard is demonstrated in Fig. 3.

Characterization of plasma native immunoreactive Met-enkephalin. Reverse-phase HPLC of standard Met-enkephalin consistently reveals two peaks of immunoreactive Met-enkephalin in both systems. The first peak coelutes with Met-enkephalin sulfoxide and the second peak with Met-enkephalin. The relative proportion of these two peaks to each other varied considerably from one study to another; it appears that Met-enkephalin is variably converted to its sulfoxide during storage, lyophilization, Porapak Q extraction, and HPLC. HPLC of rat plasma also consistently reveals two peaks of immunoreactive Met-enkephalin, the first peak also coeluting with Met-enkephalin sulfoxide and the second peak with Met-enkephalin. Fig. 4 presents an example of HPLC of rat plasma spiked with standard Met-enkephalin which had been extracted and chromatographed in duplicate. In this particular example, a third small peak intermediate between the other two was noted in each of the duplicate plasma samples; however, this small peak was not a consistent finding in other experiments.

Characterization of peptidase-derivable Met-enkephalin in plasma. Peptidase-derivable Met-enkephalin refers to cryptic Met-enkephalin present in larger peptides or proteins, or asso-

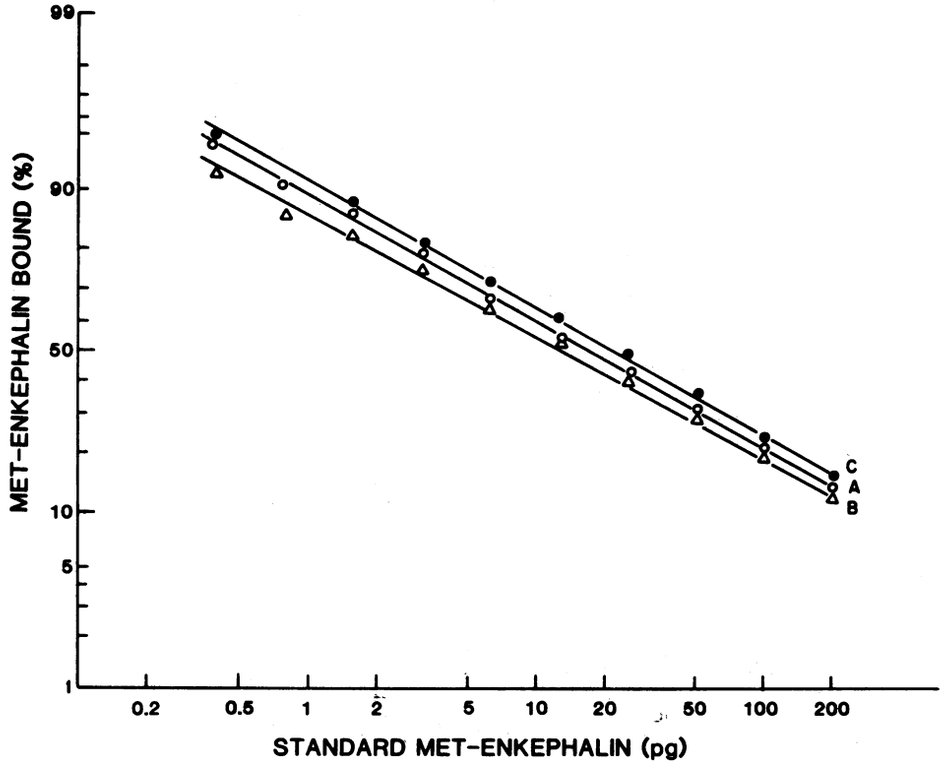

Figure 1. Recovery of Met-enkephalin standard from Porapak Q. (*A*) Standard Met-enkephalin prepared in buffer, pH 6.5, unextracted [○]. (*B*) Standard Met-enkephalin prepared in acidified plasma and buffered to pH 6.5, extracted from Porapak Q [△]. (*C*) Standard Met-enkephalin prepared in buffer, pH 6.5, extracted from Porapak Q [●]. Molar conversions for Met-enkephalin standard on the *x*-axis are 0.35, 0.9, 1.7, 3.5, 8.7, 17.4, and 35 fmol.

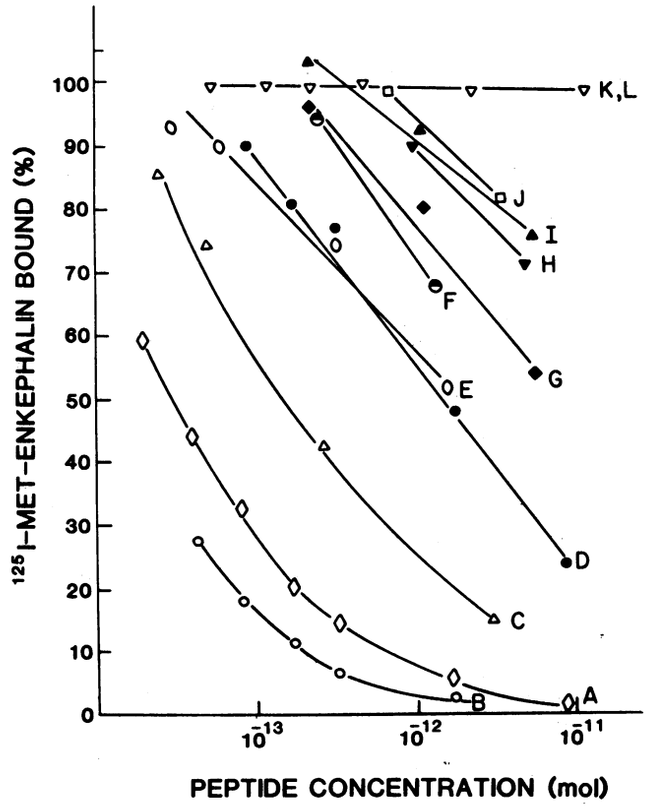

Figure 2. Cross-reactivity of enkephalin-related peptides with Met-enkephalin antiserum. (*A*) Met-enkephalin (◇). (*B*) Met-*O*-enkephalin (○). (*C*) Peptide F (△). (*D*) Leu-enkephalin (●). (*E*) Peptide E (0). (*F*) Peptide B (⊖). (*G*) Met-enkephalin-Arg^6-Phe^7 (◆). (*H*) Metorphamide (▼); (*I*) Met-enkephalin-Arg^6-Gly^7-Leu^8 (▲). (*J*) BAM-12P (□). (*K, L*) Tyr-Gly-Gly-Phe; Gly-Gly-Phe-Met (▽).

ciated with protein, and which can be generated by enzymic hydrolysis with trypsin and carboxypeptidase B. Treatment of plasma consecutively with trypsin and carboxypeptidase B results in a substantial increase in Met-enkephalin concentration. Using the hydrolytic conditions described previously (4, 17), we found an increase in plasma Met-enkephalin from 7.1±0.4 (native) to 26.1±2.1 pM (total). However, when we optimized these hydrolytic conditions, we obtained a much larger concentration of total Met-enkephalin (Table I). Currently, for generation of total Met-enkephalin in plasma we use incubation for 30 min with trypsin 1,000 μg/ml followed by incubation for 15 min with carboxypeptidase B 50 μg/ml plus trypsin inhibitor 2,500 μg/ml. Addition of cobalt chloride 0.1–50 mM to the incubation with carboxypeptidase B did not enhance the generation of Met-enkephalin. We have found subsequently that addition of cobalt chloride 1 mM to the incubation with trypsin does enhance the generation of Met-enkephalin. Identical concentrations of peptidase-derivable Met-enkephalin were obtained using plasma volumes over a range of 0.01–0.5 ml. Parallelism of total endogenous peptidase-derivable Met-enkephalin in plasma of rats, humans, and dogs with Met-enkephalin standard is demonstrated in Fig. 5.

Reverse-phase HPLC of rat plasma which had been treated sequentially with trypsin then carboxypeptidase B revealed with both systems used the presence of only two peaks of immunoreactive Met-enkephalin similar to those found for native Met-enkephalin; one peak coeluted with Met-enkephalin sulfoxide and the other coeluted with Met-enkephalin. On the other hand, reverse-phase HPLC of rat plasma followed by hydrolysis of each fraction with trypsin and carboxypeptidase B did not reveal any more Met-enkephalin or Met-enkephalin sulfoxide than was seen with measurement of the native Met-enkephalin in the corresponding fractions without hydrolysis. These data support the authenticity of this peptidase-derivable

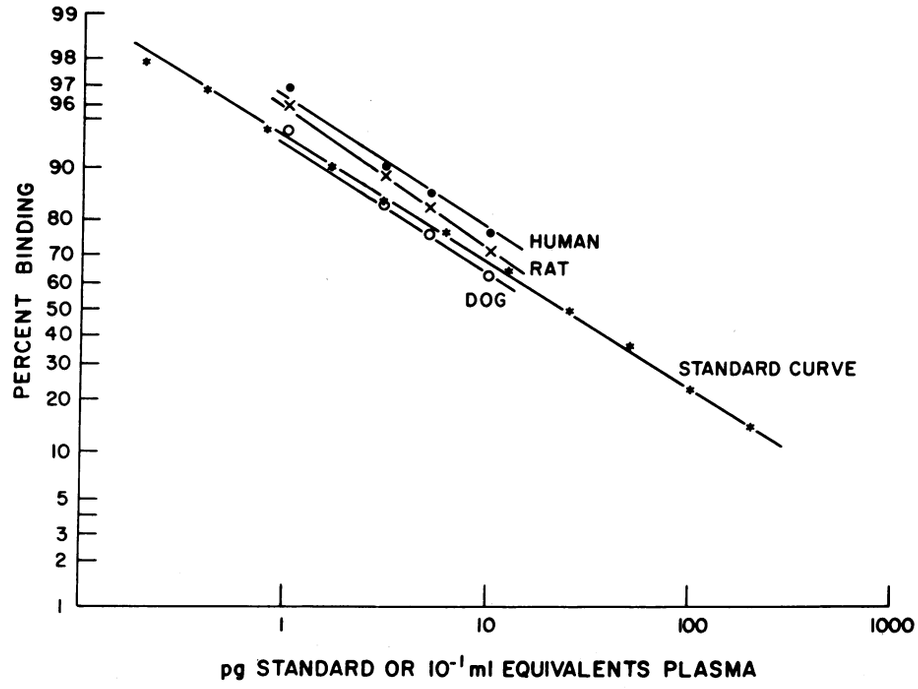

Figure 3. Parallelism of native endogenous Met-enkephalin in plasma of the rat, human, and dog with Met-enkephalin standard. Molar conversions for Met-enkephalin standard on the *x*-axis are 1.7, 17, 174, and 1743 fmol.

immunoreactive Met-enkephalin as native Met-enkephalin pentapeptide and its sulfoxide, and suggest that the peptidase-derivable Met-enkephalin is contained within a larger peptide.

Enzymic hydrolysis with trypsin then with carboxypeptidase B could be expected to release a number of intermediate peptides which would be cleaved subsequently to Met-enkephalin. We examined the effectiveness of our hydrolytic conditions to cleave a number of known proenkephalin A-derived peptides (Table II). The smaller peptides, Met-enkephalin-Arg^6-Phe^7, Met-enkephalin-Arg^6-Gly^7-Leu^8 and metorphamide, were cleaved to Met-enkephalin more readily and more completely than the larger peptides, peptides B, E, and F.

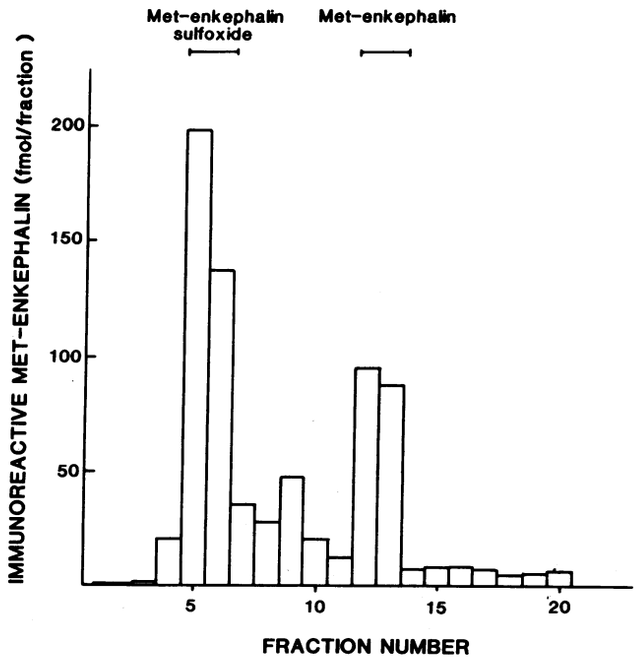

Figure 4. Reverse-phase HPLC of rat plasma (0.8 ml) spiked with standard Met-enkephalin (100 pg) and extracted on Porapak Q. Fractions represent collection for 1 min. From HPLC of unspiked plasma, it could be determined that approximately half of the immunoreactive Met-enkephalin in each fraction derived from plasma.

Guanidine hydrochloride 6 M, urea 8 M, and mercaptoethanol 1 M, were ineffective in generating from plasma the large amount of immunoreactive Met-enkephalin derived by proteolytic cleavage with trypsin and carboxypeptidase B (Table III). However, guanidine and mercaptoethanol did generate a small amount of Met-enkephalin from plasma, increasing the native concentration about 2.5-fold and 4-fold, respectively. Guanidine together with mercaptoethanol did not generate more Met-enkephalin than mercaptoethanol alone.

Plasma was filtered through a membrane with cutoff molecular mass < 30,000 D. Hydrolysis of the filtrate containing peptides with molecular mass < 30,000 D provided < 1% of the total Met-enkephalin. Met-enkephalin found after hydrolysis of the washings of the membrane which contained peptides and proteins of > 30,000 D released > 99% of the total Met-enkephalin (Table IV). We carried out a further experiment in which we filtered plasma through a membrane with cutoff molecular mass < 100,000 D. Pretreatment of plasma with guanidine plus mercaptoethanol failed to increase the amount of either native or peptidase-hydrolyzable Met-enkephalin which was filtered or the amount remaining in the retentate. Affi-Gel Blue chromatography was used to separate albumin from other proteins in the fraction containing peptides and proteins of molecular mass > 30,000 D. Enzymic hydrolysis of the supernatant obtained from centrifugation of the washed and resuspended Affi-Gel Blue pellet and, which contains mainly albumin, provided only 14% of total peptidase-derivable Met-enkephalin. In contrast, hydrolysis of the initial supernatant plus the washings from Affi-Gel Blue chro-

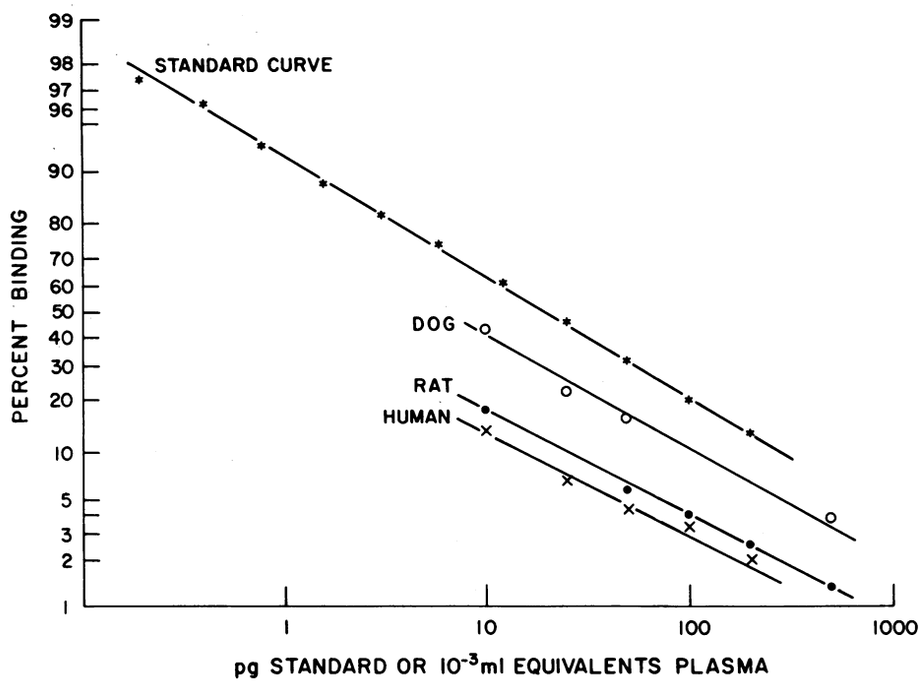

Figure 5. Parallelism of endogenous peptidase-derivable enkephalin in rat, human, and dog plasma with Met-enkephalin standard. Molar conversions for Met-enkephalin standard on the *x*-axis are 1.7, 17, 174, and 1743 fmol.

matography (containing proteins of molecular mass > 30,000 D other than albumin) revealed 86% of total Met-enkephalin. These results were confirmed by gel chromatography of plasma on Sephadex G-200 fine column. Enzymic hydrolysis with trypsin and carboxypeptidase B revealed the largest peak (65% of total) of immunoreactive Met-enkephalin in fractions 16–23 (Fig. 6 *C*) corresponding to the globulin peak of plasma protein (Fig. 6 *A*) and approximately with our thyroglobulin standard, molecular mass 669,000 D (Fig. 6 *B*). A second large Met-enkephalin peak (22% of total) was found in fractions 32–37 corresponding to the albumin standard (67,000 D) and to the albumin peak of plasma protein. A third small peak (11%) of Met-enkephalin in fractions 28–31 corresponded to a shoulder on the front of the albumin peak of plasma proteins and to protein of molecular mass 100,000–150,000 D. It is unclear whether two other peaks of Met-enkephalin were present on the back shoulder of the larger globulin peak between 250,000 and 500,000 D. These findings of multiple high molecular mass protein peaks which release immunoreactive Met-enkephalin upon peptidic hydrolysis were replicated on Sephadex G-200 chromatography on three separate occasions.

Table II. Generation of Peptidase-derived Met-enkephalin from Enkephalin-related Peptides

Peptide	1 ng	10 ng
	%	
Met-enkephalin-Arg^6-Phe^7	12	42
Met-enkephalin-Arg^6-Gly^7-Leu^8	26	36
Metorphamide	50	43
BAM-12P	24	33
Peptide B	3	1
Peptide E	4	4
Peptide F	4	7
D-Ala^2-Met-enkephalinamide	0	0

Data are presented as percent of peptide converted to immunoreactive Met-enkephalin.
Either 1 or 10 ng of peptide was incubated sequentially with trypsin 10 μg and carboxypeptidase B 1 μg, and the medium assayed for Met-enkephalin. The data were corrected for recovery by incubation and assay of Met-enkephalin standard.

Bioactivity of peptidase-derivable Met-enkephalin. Plasma extract which had been hydrolyzed with trypsin and carboxypeptidase B to release total Met-enkephalin and extracted on Porapak Q was run on reverse-phase HPLC. Fractions representing Met-enkephalin and Met-enkephalin sulfoxide were studied individually in a mouse vas deferens assay to quantitate bioactive Met-enkephalin. The responses of authentic Met-enkephalin pentapeptide obtained from aliquots representing 11.2 and 22.4 ml of plasma are compared with the responses to Met-enkephalin standard in Fig. 7. These data provided an estimate for plasma concentration of bioactive peptidase-derivable Met-enkephalin of 3,973 pM, very comparable to our estimates of plasma concentration of immunoreactive Met-enkephalin. Met-enkephalin sulfoxide isolated by HPLC from the enzymically hydrolyzed plasma pool appeared to be at least as potent in this bioassay system as the similarly isolated Met-enkephalin. Furthermore, the effects of both plasma peptidase-derivable Met-enkephalin and its sulfoxide to inhibit electrically-induced contraction of mouse vas deferens were reversed completely by addition of naloxone to the perfusion bath at a time when the peptides were maximally active.

Plasma Met-enkephalin responses to restraint stress. Time courses of plasma native and peptidase-derivable Met-enkephalin responses to restraint stress are shown in Fig. 8; each point represents the mean of 2–10 different experiments with 7–11 rats in each experiment. Plasma native Met-enkephalin level increased from a basal level of 9.6±0.8 pM (mean±SEM) to a

Table III. Generation of Immunoreactive Met-enkephalin by Protein-disrupting Agents

	Met-enkephalin concentration					
	Native	Trypsin plus carboxypeptidase B	Urea, 8 M	Guanidine, 6 M	Mercaptoethanol, 1 M	Guanidine plus mercaptoethanol*
				pM		
Experiment 1	29	3.779	35	82	—	—
	40	4,960	44	161	—	—
Experiment 2	31	2,395	—	65	172	—
	43	2,468	—	72	155	—
Experiment 3	47	1,987	—	—	—	137
	27	1,832	—	—	—	174

* Guanidine 6 M plus mercaptoethanol 1, 5, or 10 M gave identical results.

peak of 30.2±10.4 pM ($P < 0.01$) at 0.5 min of restraint and remained elevated only briefly. In spite of continuation of stress, plasma level of native Met-enkephalin declined to 18 pM by 2 min, 15 pM by 2.5 min, and 12.5 pM by 5 min, and it was not different from basal level at this time. Prolonged restraint produced a second peak of plasma native Met-enkephalin at 30 min of stress. Plasma level of Met-enkephalin increased from 9.2±1.7 pM at 20 min of restraint to 27.9±4.0 pM at 30 min ($P < 0.01$). 10 min after the end of 30 min of restraint stress, plasma native Met-enkephalin level was no different from that seen in control animals. Immediately after the 60 min period of restraint, rats were decapitated. Plasma concentrations of both native (8.0±1.2 pM) and peptidase-derivable (5,294±655 pM) Met-enkephalin in trunk blood at this time were identical to those in blood taken from the arterial cannula at 60 min of restraint.

Nonstressed control animals did not show any significant changes in plasma level of native Met-enkephalin during the entire period of the experiments. Since withdrawal of large volumes of blood may result in sympathoadrenal activation, it was necessary to demonstrate that our blood sampling protocols did not increase plasma Met-enkephalin concentration. The effect of repeated blood sampling on plasma Met-enkephalin levels was examined using several protocols in these experiments. Neither frequent withdrawals of a small volume (0.26 ml) for a total withdrawal of 2.6 ml of blood over 10 min in 300-g rats nor less frequent withdrawals of larger volumes (0.5–0.8 ml) for a total withdrawal of 3.5 ml of blood over 30 min resulted in elevation of plasma native Met-enkephalin.

Similar to native Met-enkephalin, plasma peptidase-derivable Met-enkephalin response was maximal at 0.5 min of restraint, increasing from a basal level of 3,450 to a peak of 5,260 pM, but this response was somewhat more prolonged than that for native Met-enkephalin. Plasma peptidase-derivable Met-enkephalin returned to basal level by 7 min. Plasma peptidase-derivable Met-enkephalin again showed an elevated level at 30 min of restraint stress (from 3,193±49 to 4,289±76 pM), but this response was much less than the initial response at 0.5 min of stress. Plasma peptidase-derivable Met-enkephalin levels in control unstressed animals did not show any statistically significant differences during the experiment. Also similar to native Met-enkephalin, our blood sampling protocols did not produce changes in plasma concentration of total Met-enkephalin.

Time courses of plasma norepinephrine and epinephrine responses to restraint stress are shown in Fig. 9; each value is the mean of one to eight different experiments. Plasma norepinephrine increased from a basal level of 1.45±0.15 to a peak of 5.19±0.39 nM, also at 0.5 min of restraint. The peak plasma level of norepinephrine lasted for the next 0.5 min of stress ($P < 0.01$), then declined slowly but still remained significantly ($P < 0.05$) elevated to the end of the 30 min of restraint. Plasma epinephrine increased from a basal level of 1.68±0.24 to a peak of 12.69±0.64 nM, also at 0.5 min of restraint, then decreased to 6.42±0.81 nM by 2 min and declined slowly to 4.9±1.0 nM by 30 min of stress; this concentration was still elevated above basal level ($P < 0.01$). Control animals did not show any significant changes in the plasma concentrations of norepinephrine and epinephrine during experiments.

Since it has been demonstrated that repeated administration of an identical stressor resulted in adaptation of catecholamine responses (18), it was of interest to study possible

Table IV. Distribution of Total Peptidase-derived Met-enkephalin Associated with Proteins in Rat Plasma

Separation technique	Molecular mass	Met-enkephalin concentration	Recovery
	D	*pM*	%
Filtration through Amicon Diaflo	<30,000	38	
Ultra-filters PM30 membrane	>30,000	4,706	95
Affi-gel blue chromatography	Albumin (67,000)	627	
	>67,000	4,009	93

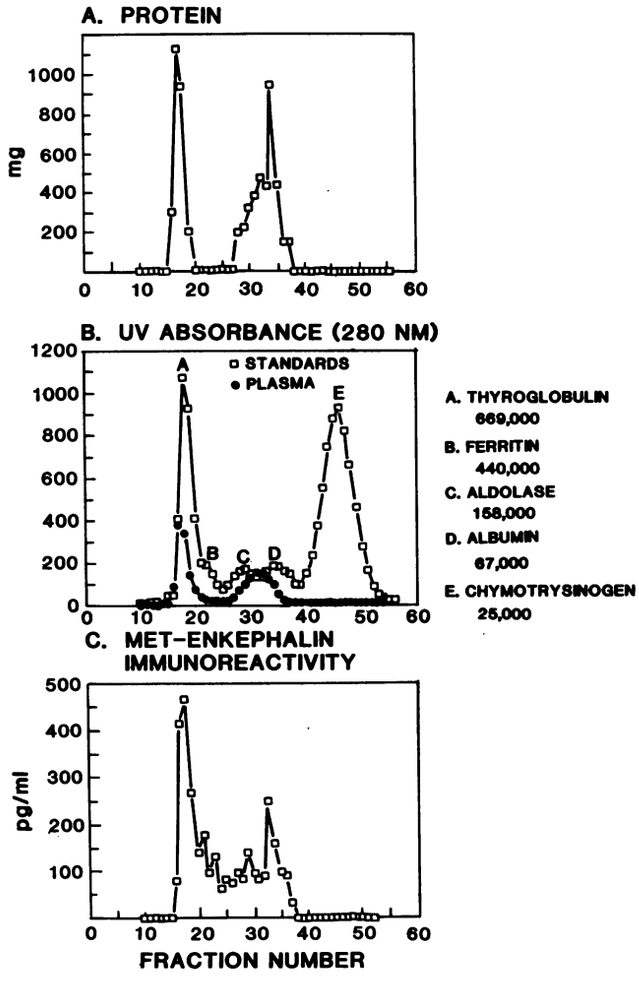

Figure 6. Chromatograms of rat plasma and protein standards on Sephadex G-200 fine column. (*A*) protein (Lowry), mg/100 ml of plasma; (*B*) ultraviolet absorbance of protein standards and plasma; (*C*) Met-enkephalin immunoreactivity, pg/ml.

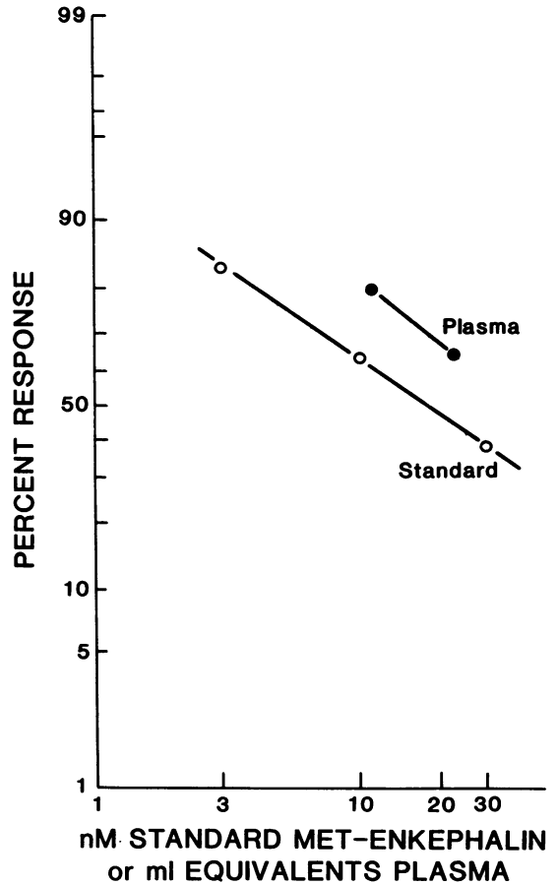

Figure 7. Bioactivity of plasma peptidase-derivable Met-enkephalin. The effects of standard Met-enkephalin and Met-enkephalin generated by enzymic hydrolysis of plasma to inhibit electrically-induced contraction of mouse vas deferens in vitro are compared.

changes in the plasma native and peptidase-derivable Met-enkephalin responses to repeated exposure to restraint stress. Thus, rats were exposed to restraint stress daily for 30 min, and plasma Met-enkephalin and catecholamine responses were measured. Plasma native and peptidase-derivable Met-enkephalin responses to initial, acute 30 min restraint on days 1 and 7 after 1 wk of daily exposure to 30 min restraint are shown in Fig. 10. Acute restraint stress increased rapidly the plasma level of native Met-enkephalin from a basal value of 8.9±0.8 to 30.7±3.1 pM at 1 min ($P < 0.01$). As shown previously in time course experiments, plasma level of native Met-enkephalin declined after a transient elevation, and by 10 min of restraint was not different from basal level. Also, prolonged stress to 30 min caused a second increase of plasma native Met-enkephalin level up to 21.3±2.2 pM at the end of restraint. Repeated daily exposure to 30 min of restraint stress for 1 wk resulted in complete loss of the plasma native Met-enkephalin response to acute restraint.

Acute restraint stress increased plasma concentrations of peptidase-derivable Met-enkephalin from a basal level of 3,337±127 to a peak of 4508±293 pM at 1 min ($P < 0.01$). Met-enkephalin remained elevated at 15 min of stress (4,127±270 pM, $P < 0.05$) and again increased further at 30 min of stress to 4,480±239 pM. The basal level of plasma peptidase-derivable Met-enkephalin on day 7 was elevated, but this increase was not statistically significant. Rats exposed to restraint stress for 30 min daily for 1 wk showed a tendency on day 7 to increase plasma levels of peptidase-derivable Met-enkephalin, but the increases were not statistically significant. Plasma levels of peptidase-derivable Met-enkephalin in the control unstressed animals did not show any changes during blood sampling, either on day 1 or 7.

Plasma epinephrine and norepinephrine responses to acute restraint stress, initially and after 1 wk of daily stress, are compared in Fig. 11. Acute 30 min restraint on day 1 produced a maximal increase in plasma concentration of epinephrine from a basal level of 0.9±0.27 to a peak of 10.07±1.22 nM ($P < 0.01$) at 1 min of stress. As noted above, plasma epinephrine declined somewhat but remained significantly elevated above basal level throughout the period of acute restraint. After 1 wk of daily restraint stress, the plasma epinephrine response to restraint on day 7 was blunted when compared to the response on day 1, but the epinephrine response remained statistically significant at 1 and at 30 min of stress. Plasma norepinephrine response to initial restraint was similar to that described in the experiments above. After a maximal increase seen at 1 min, plasma norepinephrine had decreased by 15 min but was still

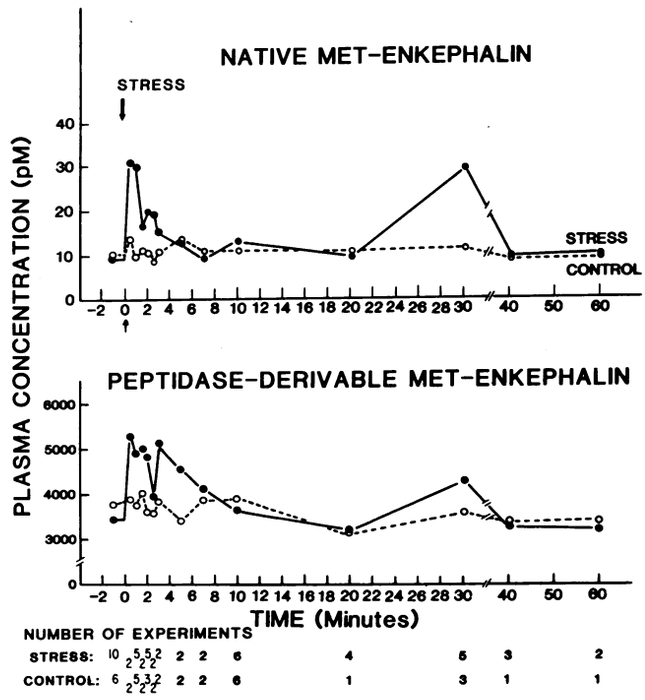

Figure 8. Plasma immunoreactive Met-enkephalin responses to restraint stress. Data from 11 experiments were combined; numbers indicate numbers of experiments at each time point with 7–11 rats per experiment. (○) Control; (●) restraint. Data were analyzed by ANOVA and Duncan's multiple range test. After stress, statistically significant differences from baseline were present at 0.5, 1, 2, 2.5, and 30 min for native and at 0.5, 1, 1.5, 2, 3, 5, and 30 min for peptidase-derivable Met-enkephalin. No significant differences were noted in control unstressed rats.

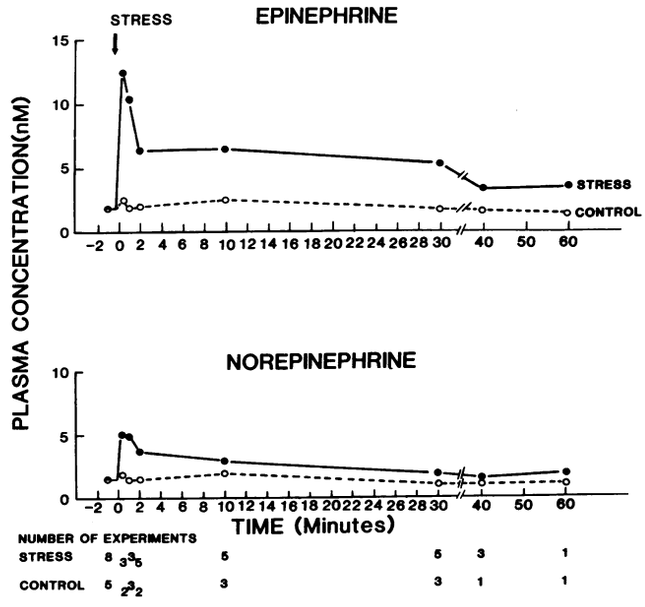

Figure 9. Plasma catecholamine responses to restraint stress. Data from eight experiments were combined; numbers indicate numbers of experiments at each time point. (○) control; (●) restraint. Data were analyzed by ANOVA and Duncan's multiple range Test. After stress, statistically significant differences from baseline were present at 0.5, 1, 2, 10, and 30 min for both native and peptidase-derivable Met-enkephalin. No significant differences were noted in control unstressed rats.

significantly elevated above baseline at the end of the 30-min stress. In contrast to epinephrine, on day 7 plasma norepinephrine did increase significantly above the basal concentration but did not remain elevated throughout the 30-min period of stress.

Discussion

We have described a very sensitive method for measurement in plasma (and other biological fluids) of native and peptidase-derivable Met-enkephalin. This method employs a commercial antiserum and a tracer radiolabeled either commercially or in our laboratory. Radioimmunoassays for Met-enkephalin described previously have reported a wide range for basal plasma concentration (8). This variation may relate to differences in methods of blood collection, in methods of extraction of peptide from plasma, and in antisera.

Previously, we had found that published methods of blood collection for Met-enkephalin were not optimal, and we introduced the use of citrate to prevent degradation of the pentapeptide (12). We found citrate to be more effective in this regard than the specific aminopeptidase inhibitor, bestatin, and subsequently we reduced the final concentration of citrate from 23 to 17 mM to prevent hemolysis which occurs with citrate in some blood, particularly sheep blood. We also use aprotinin to prevent degradation of large precursor peptides to Met-enkephalin. In unpublished studies, we found that addition of EDTA did not affect the final concentration of Met-en-

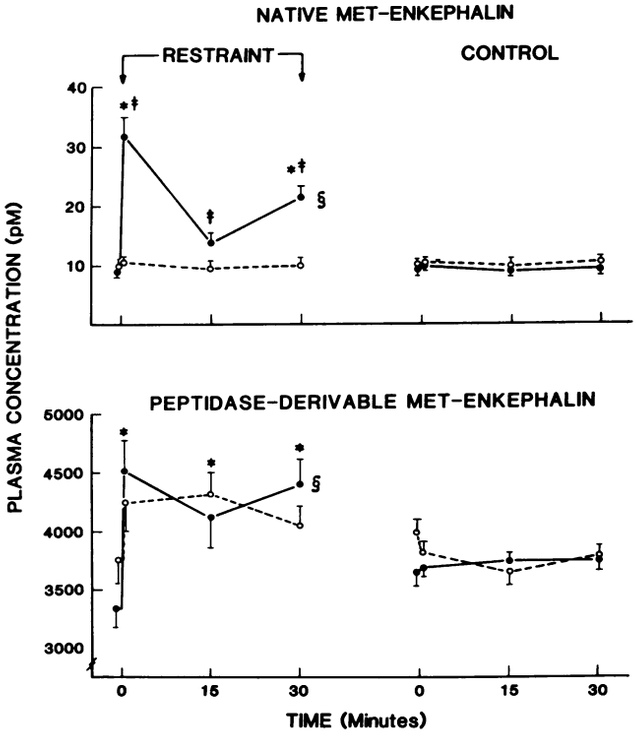

Figure 10. Plasma native and total peptidase-derivable Met-enkephalin responses to initial (day 1, ●) and repeated daily (day 7, ○) 30-min restraint stress ($\bar{x}\pm$SEM). *Significant difference at $P < 0.05$ from basal concentration at time −1 min. ‡Significant difference at $P < 0.05$ from concentration at corresponding day 7 time point. §Significant change at $P < 0.01$ for the overall curve from baseline.

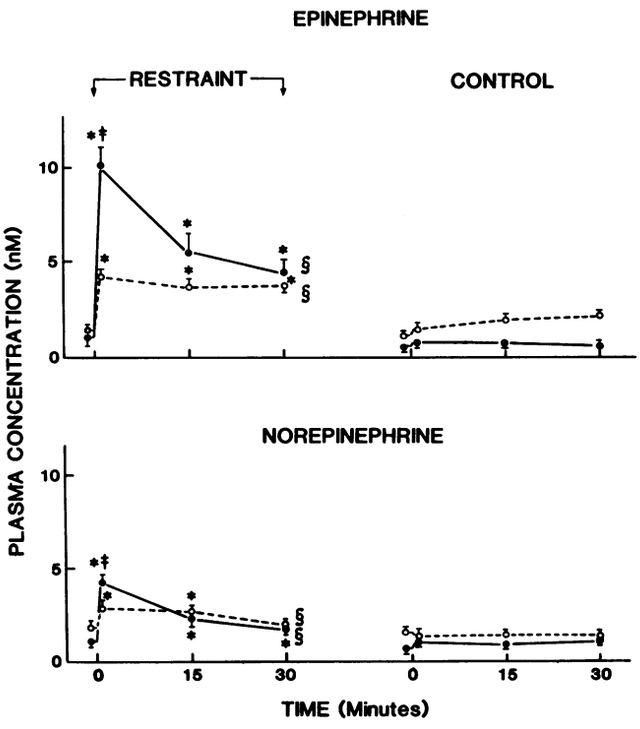

Figure 11. Plasma epinephrine and norepinephrine responses to initial (day 1, ●) and repeated daily (day 7, ○) 30-min restraint stress ($\bar{x}\pm$SEM). *Significant difference at $P < 0.05$ from basal concentration at time −1 min. ‡Significant difference at $P < 0.05$ from concentration at corresponding day 7 time point. §Significant change for the overall curve from baseline at $P < 0.01$.

kephalin detected. Also, heparin did not affect immunoreactivity of native Met-enkephalin. However, collection of blood with EDTA or heparin in addition to citrate and aprotinin resulted in lower concentrations of immunoreactive peptidase-derivable Met-enkephalin than we found in their absence. Acidification of plasma is an important step in maintaining the stability of Met-enkephalin in plasma.

Porapak Q, a porous copolymer of styrene and ethylvinylbenzene cross-linked with divinylbenzene, has been reported to be effective in absorbing enkephalins (12, 19). We found Porapak Q very effective in extracting Met-enkephalin from plasma of the human, rat, dog, and sheep. We (unpublished) and others (20) have found C_{18} resin much less effective; also, octadecylthrichlorosilane provides poor recovery of Met-enkephalin from plasma (21). Acid-acetone extraction has been used effectively when large volumes of plasma or very high concentrations of Met-enkephalin are available (22).

Several assays for Met-enkephalin have employed antisera generated against Met-*O*-enkephalin sulfoxide which do not cross-react with Met-enkephalin but require oxidation of samples before assay. In our assay, Met-*O*-enkephalin sulfoxide was even more immunoreactive than Met-enkephalin. The only other peptide which showed cross-reactivity of note (~ 20%) was Peptide F. Kraemer et al. (23) reported plasma levels of peptide F to be considerably higher than those of Met-enkephalin in human plasma. Similar comparison in rat plasma has not been reported.

Incubation of adrenal cell culture medium (4) or tissue (24) with the peptidases, trypsin and carboxypeptidase B, releases Met-enkephalin from proenkephalin A and from a number of peptidic intermediates. Peptidic hydrolysis of large peptides and proteins in plasma may yield a number of biologically active peptides (8, 10, 22, 25, 26). Thus, extracellular and even intravascular production of peptides by limited proteolytic cleavage from secreted precursors may represent a physiologic mechanism of origin of bioactive peptides. Proteases such as trypsin may act in a manner similar to an enzyme such as kallikrein which physiologically processes kininogen to bradykinin-related peptides (27, 28). Potential cleavage sites and proteolytic mechanisms have been reviewed elsewhere (10, 25, 27, 28). As we noted above, incubation of plasma with concentrations of trypsin and carboxypeptidase B used in those published studies increased the yield of immunoreactive Met-enkephalin by about threefold. When we increased the concentration of these peptidases to a more optimal level, we increased the yield of Met-enkephalin from plasma greatly to ~ 350-fold. Furthermore, we have found that acid hydrolysis using lyophilization of plasma in strong acid also yields large amounts of immunoreactive Met-enkephalin in plasma (29). In contrast to the report of Singer et al. (22), we were unable to increase plasma concentrations of Met-enkephalin by incubation with pepsin. Apparently, these investigators were unable to generate increases in Met-enkephalin concentration in plasma using trypsin and carboxypeptidase B, although it seems that they did not use trypsin inhibitor together with carboxypeptidase B and they used much lower concentrations of these peptidases. Also, the findings of Singer et al. (22) of pepsin-induced generation of immunoreactive Met-enkephalin from plasma may relate in part to their incubation and lyophilization of plasma at low pH. Finally, their use of acid-acetone extraction of plasma may alter the yield of Met-enkephalin from plasma. Recently, Giraud et al. (30) described generation of Met-enkephalin-Arg^6-Phe^7 from rat plasma using pepsin. Although the antiserum used in our studies does not cross-react significantly with Met-enkephalin-Arg^6-Phe^7, we have found that trypsin followed by carboxypeptidase B will cleave a significant portion of this heptapeptide to Met-enkephalin (Table II).

Neither arylsulfatase nor β-glucuronidase generated Met-enkephalin from plasma (29), suggesting that Met-enkephalin does not circulate in significant concentration as a conjugate of either sulfate or glucuronide. Incubation of plasma with guanidine hydrochloride generated a small amount of Met-enkephalin, suggesting that this small amount of Met-enkephalin might circulate in noncovalent association with albumin. Also, generation of a small amount of native Met-enkephalin by incubation of plasma with mercaptoethanol suggests that some Met-enkephalin is linked to protein by disulfide bonds. However, none of guanidine hydrochloride, urea, or mercaptoethanol generated the large amount of Met-enkephalin seen following enzymic cleavage with trypsin and carboxypeptidase B, suggesting further that this total peptidase-derivable Met-enkephalin in plasma is not present simply in noncovalent or disulfide bonding to protein. Also, pretreatment of plasma with guanidine plus mercaptoethanol, before their filtration through a membrane with cutoff of molecular mass < 100,000 D did not result in an increase in Met-enkephalin which could be released by peptidic hydrolysis; these data suggest that this peptidase-derivable Met-enkephalin is not present simply in proenkephalin A bound noncovalently to larger proteins. Furthermore, it seems likely that the Met-enkephalin sequence is present near the amino or carboxy terminus of the protein or in a loop sequence since it is so readily released by trypsin, and

it is probably on the surface of the protein since it does not require denaturation for its release.

We demonstrated on mouse vas deferens that both peptidase-derivable Met-enkephalin and its sulfoxide had naloxone-reversible bioactivity comparable to that of synthetic Met-enkephalin. These data provided further confirmation for our radioimmunoassay and HPLC findings that this peptidase-derivable Met-enkephalin was indeed authentic Met-enkephalin pentapeptide. Previously, we had also shown that total immunoreactive Met-enkephalin derived in the same manner by enzymic hydrolysis with trypsin and carboxypeptidase B from brain tissue was bioactive (31). The data also suggest that this pool of peptidase-derivable Met-enkephalin in plasma may provide a very significant source of bioactive Met-enkephalin.

Chromatography of rat plasma on Sephadex G-200 yielded two or three major protein peaks, one in the molecular mass range of 600,000 D and another at ~ 67,000 D with a shoulder running up to 150,000 D. After Sephadex G-200 chromatography, digestion of fractions with trypsin and carboxypeptidase B yielded a large peak of immunoreactive Met-enkephalin at a position corresponding to globulins or other proteins of molecular mass 600,000–700,000 D, a smaller peak corresponding to albumin and several even smaller and questionable peaks in between. Boarder et al. (32) found only small amounts of Met-enkephalin-like material associated with peptides of > 12,000 D in human plasma. Singer et al. (22) found in pepsin-treated plasma a single peak of Met-enkephalin immunoreactivity corresponding to a protein of molecular mass ~ 65,000 D. We have found that a significant quantity of immunoreactive Met-enkephalin can be generated from bovine serum albumin by sequential incubation with trypsin and carboxypeptidase B. Shen and Lindberg (33) generated from rat serum albumin a peptide which was immunoreactive in their Met-enkephalin RIA, but which was more hydrophilic and had a different pI from authentic Met-enkephalin. Our data with both Sephadex G-200 chromatography and with Affi-Gel Blue chromatography suggested that 14–22% of the immunoreactive Met-enkephalin which we generated from plasma derived from albumin or a protein of similar molecular weight. Thus, it appears that trypsin (in our hands) or pepsin (in the hands of other investigators [22, 33] but not in our hands) are able to lyse albumin or a closely related protein to yield immunoreactive Met-enkephalin. The major peak of immunoreactive Met-enkephalin which we obtained upon sequential digestion with trypsin and carboxypeptidase B of fractions from Sephadex G-200 chromatography of rat plasma was not albumin, but rather a protein of the size of a globulin with molecular mass > 600,000 D. In any case, and while it is not possible to entirely rule out the possibility that preproenkephalin A is bound to a large protein, it is clear from our studies and from those of others (22, 30, 33) that largely the plasma protein precursors for peptidase-derivable Met-enkephalin and related peptides differ structurally and chemically from proenkephalin A.

Possenti et al. (34) provided evidence that enkephalins are bound to protein in plasma. However, the type of binding remains obscure. From the conditions required to generate the large amounts of enkephalin-related peptides from plasma in our studies and in those of others (22, 30, 33), it seems probable that this immunoreactive Met-enkephalin is either an intrinsic part of the protein molecules or is very strongly bound to proteins. Renin is a 40,000-D molecular mass protein which may also exist in plasma as an 800,000-D protein (35), probably in complex with a number of binding proteins including α_2-macroglobulin (36). It is possible that Met-enkephalin or preproenkephalin A also are bound strongly in a specific conformation with plasma proteins; immunoelectrophoretic identification of these binding proteins might be facilitated by unfolding and refolding of labelled preproenkephalin A by guanidine. Differences between the generation of another neuropeptide, neurotensin, from brain cells and neurotensin-related peptides from plasma have been discussed by Carraway et al. (26). Analogies may exist for neuronal Met-enkephalin and the peptidase-derivable immunoreactive Met-enkephalin generated from plasma. Thus, native Met-enkephalin processed from proenkephalin A and stored within vesicles in sympathetic nerves or chromaffin cells is released upon appropriate stimulation. On the other hand, Met-enkephalin or a closely related immunoreactive peptide present in another large precursor form in plasma may undergo rapid proteolysis similar to that seen for generation of bradykinin or angiotensin.

Previous studies have documented the co-secretion of immunoreactive Met-enkephalin and catecholamines from chromaffin cells in culture (3, 4), from isolated perfused adrenal glands (6, 7), and from dog adrenals in vivo (5). However, plasma Met-enkephalin responses to stimuli have been documented in relatively few studies (cf. 8, 37–39). In this study, we describe in rats consistent rapid increases in plasma immunoreactive native Met-enkephalin in response to restraint, a psychological stress. Plasma native Met-enkephalin peaked in parallel with the increases in plasma epinephrine and norepinephrine. Thereafter, there was a divergence in the plasma concentrations of Met-enkephalin and catecholamines during the period of restraint stress. Plasma Met-enkephalin showed a biphasic response to 30 min of restraint. A somewhat similar phasic response pattern has been reported for plasma epinephrine and norepinephrine in response to hemorrhage, with an initial peak within ~ 5 min and a second peak at 20–30 min (40). Also, the apparently rapid clearance of the large molecular mass proteins which yield the peptidase-derivable Met-enkephalin may relate to increased secretion of large protein for only a very brief period after onset of stress; then, the apparent clearance of the large protein would in fact reflect clearance of Met-enkephalin. Whether these phases of Met-enkephalin response represent secretion from different compartments in the same tissue similar to that seen with insulin (41) or secretion from different tissues remains unknown. It seems probable that the brief duration of the initial peak of plasma Met-enkephalin induced by restraint stress is a result of a central nervous system regulatory mechanism rather than of a limitation in Met-enkephalin pool size, since the more severe stress of immobilization produced a prolonged elevation of plasma Met-enkephalin (42).

The adrenal medulla has seemed to most investigators to represent the most probable source of circulating Met-enkephalin, and we have found that in dogs adrenal secretion rate of native Met-enkephalin increases briskly in response to insulin-induced hypoglycemia (8) or nicotine (43). On the other hand, the adrenomedullary concentration of Met-enkephalin is considerably lower in rats than in other species (44). We have carried out a study to determine the sympathetic nerve and adrenomedullary contributions to the plasma Met-enkephalin increases seen in response to restraint in rats (45). Chemical sympathectomy with guanethidine, but not adrenal

demedullation, prevented the plasma native Met-enkephalin response to restraint stress. These data suggest that in rats the restraint stress-induced increase in plasma native Met-enkephalin is derived largely from sympathetic nerves and not from the adrenal medulla. It is well known that plasma catecholamine concentrations in trunk blood after decapitation are markedly higher than those in blood taken from an indwelling cannula (18), presumably because of massive adrenomedullary discharge associated with decapitation. In contrast, plasma Met-enkephalin concentration does not appear to be affected by decapitation in rats. This may also argue against the adrenal medulla as a major source of circulating Met-enkephalin in rats. We have been unable to reduce basal plasma Met-enkephalin with chemical sympathectomy, adrenal demedullation, hypophysectomy, or evisceration (Barron, B., and G. R. Van Loon, unpublished results). A small amount of circulating Met-enkephalin may derive from median eminence since levels are higher in hypothalamohypophyseal portal blood than in peripheral blood (Van Loon, G. R., and P. Plotsky, unpublished results).

Peptidase-derivable Met-enkephalin showed multiphasic response patterns to restraint stress which paralleled those for native Met-enkephalin, although the concentration varied from 150- to 375-fold higher than for native Met-enkephalin. The origin of the stress-induced increase in plasma peptidase-derivable Met-enkephalin is more difficult to understand than is the origin of the stress-induced increase in native Met-enkephalin.

Repeated exposure to an identical stressor results in adaptive loss of the plasma catecholamine responses to stress (18). Thus, it was of considerable interest to determine whether the plasma Met-enkephalin responses to repeated exposure to stress showed a similar adaptation. Sympathoadrenal release of Met-enkephalin showed rapid adaptation of response; after 6 d of daily 30-min restraint stress, there were no longer plasma native or peptidase-derivable Met-enkephalin responses to restraint. Although the plasma epinephrine and norepinephrine responses to restraint were blunted after 6 d of daily 30-min restraint stress, they remained significant. This suggests to us that adaptation of the sympathetic nerve responses to a psychological stress occurs more readily than adaptation of the adrenomedullary responses. A somewhat different pattern of adaptation of plasma Met-enkephalin responses was seen with immobilization stress, and both native and peptidase-derivable Met-enkephalin showed cross-tolerance to hemorrhagic or electric footshock stress when adapted to immobilization (42). It remains unknown whether adaptation of plasma Met-enkephalin responses to restraint or immobilization stress is mediated in brain, in the peripheral sympathoadrenal Met-enkephalin pool, or in both. A number of response patterns have been noted during adaptation of sympathoadrenal responses to chronic stress (46). These include increased biosynthetic and storage capacity, elevated basal activity, attenuated responsiveness to homotypic stimulation and similar or potentiated responsiveness to novel stimulation. However, no clear hypothesis has emerged to unify these observations into a clear explanation of adaptation of the sympathoadrenal responses to repeated stress.

The function of circulating Met-enkephalin remains elusive, but roles for opioid peptides in neuroendocrine (2) and cardiovascular regulation (37, 47, 48) as well as modulation of immune function (49) have been proposed. Although the plasma concentrations of native Met-enkephalin (5–50 pM) appear rather low to effectively mediate humoral responses, nanomolar to micromolar concentrations of peptidase-derivable Met-enkephalin demonstrated in plasma, and changes in plasma concentration of this peptidase-derivable Met-enkephalin in response to physiologic stimuli, suggest a functional role.

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References

1. Schultzberg, M., J. M. Lundberg, T. Hokfelt, L. Terenius, J. Brandt, R. P. Elde, and M. Goldstein. 1978. Enkephalin-like immunoreactivity in gland cells and nerve terminals of the adrenal medulla. *Neuroscience.* 3:1169–1186.

2. Kiritsy-Roy, J. A., and G. R. Van Loon. 1988. Endogenous opioid peptides in neuroendocrine regulation. *In* Clinical Neuroendocrinology. R. Collu, G. M. Brown, and G. R. Van Loon, editors. Blackwell Scientific Publications, Boston. 491–514.

3. Livett, B. G., D. M. Dean, L. J. Whelan, S. Udenfriend, and J. Rossier. 1981. Co-release of enkephalin and catecholamines from cultured adrenal chromaffin cells. *Nature (Lond.).* 289:317–319.

4. Wilson, S. P., K. J. Chang, and O. H. Viveros. 1982. Proportional secretion of opioid peptides and catecholamines from adrenal chromaffin cells in culture. *J. Neurosci.* 2:1150–1156.

5. Hanbauer, I., G. D. Kelly, L. Saiani, and H.-Y. T. Yang. 1982. [Met^5]-enkephalin-like peptides of the adrenal medulla: release by nerve stimulation and functional implications. *Peptides.* 3:469–473.

6. Asada, N., T. Saito, T. Kanno, K. Nakao, T. Yoshimasa, and H. Imura. 1983. Parallel time courses of acetylcholine-induced releases of catecholamine and enkephalin-like immunoreactive peptides from perfused dog adrenal glands. *Biomed. Res.* 4:413–416.

7. Chaminade, M., A. S. Foutz, and J. Rossier. 1983. Co-release of enkephalins and precursors with catecholamine by the perfused cat adrenal in-situ. *Life Sci.* 33(Suppl.)1:21–24.

8. Van Loon, G. R., K. Pierzchala, L. V. Brown, and F. A. Bobbitt. 1987. Plasma free and cryptic Met-enkephalin responses to stress. *In* Synaptic Transmitters and Receptors. S. Tucek, editor. Academia, Prague. 355–364.

9. Stern, A. S., B. N. Jones, J. E. Shiveley, S. Stein, and S. Udenfriend. 1981. Two adrenal opioid polypeptides: proposed intermediates in the processing of proenkephalin. *Proc. Natl. Acad. Sci. USA.* 78:1962–1966.

10. Loh, Y. P., M. J. Brownstein, and H. Gainer. 1984. Proteolysis in neuropeptide processing and other neural functions. *Annu. Rev. Neurosci.* 7:189–222.

11. Snyder, S. H., and L. D. Fricker. 1984. Enkephalin biosynthesis: focus on enkephalin convertase. *In* Opioids Past, Present, and Future. J. Hughes, H. O. J. Collier, M. J. Rance, and M. B. Tyers, editors. Taylor & Francis, London. 193–205.

12. Aloyo, V. J., S. A. Mousa, and G. R. Van Loon. 1986. Stabilization of methionine-enkephalin in human and rat blood. *Life Sci.* 39:21–28.

13. Sole, M. J., and M. N. Hussain. 1977. A single, specific radioenzymatic assay for the simultaneous measurement of picogram quantities of norepinephrine, epinephrine, and dopamine in plasma and tissue. *Biochem. Med.* 18:301–307.

14. Henderson, G., J. Hughes, and H. W. Kosterlitz. 1972. A new

example of a morphine-sensitive neuro-effector junction: adrenergic transmission in the mouse vas deferens. *Br. J. Pharmacol.* 46:764–766.

15. Van Loon, G. R., N. M. Appel, and D. Ho. 1981. Beta-endorphin-induced increases in plasma epinephrine, norepinephrine, and dopamine in rats: inhibition of adrenomedullary responses by intracerebral somatostatin. *Brain Res.* 212:207–214.

16. De Souza, E. B., and G. R. Van Loon. 1982. Stress-induced inhibition of the plasma corticosterone response to a subsequent stress in rats: a nonadrenocorticotropin-mediated mechanism. *Endocrinology.* 110:23–33.

17. Smith, R., A. Grossman, R. Gaillard, V. Clement-Jones, S. Ratter, J. Mallinson, P. J. Lowry, G. M. Besser, and L. H. Rees. 1981. Studies on circulating Met-enkephalin and B-endorphin: normal subjects and patients with renal and adrenal disease. *Clin. Endocrinol.* 15:291–300.

18. Kvetnansky, R., S. Nemeth, M. Vigas, Z. Oprsalova, and J. Jurcovicova. 1984. Plasma catecholamines in rats during adaptation to intermittent exposure to different stressors. *In* Stress. The Role of Catecholamines and Other Neurotransmitters. E. Usdin, R. Kvetnansky, and J. Axelrod, editors. Gordon and Breach Science Publishers, Inc., New York. 537–562.

19. Vogel, Z., and M. Altstein. 1977. The adsorption of enkephalin to porous polystyrene beads: a simple assay for enkephalin hydrolysis. *FEBS (Fed. Eur. Biochem. Soc.)* Lett. 80:332–336.

20. Yoshimasa, T., K. Nakao, S. Li, Y. Ikeda, M. Suda, M. Sakamoto, and H. Imura. Plasma methionine-enkephalin and leucine-enkephalin in normal subjects and patients with pheochromocytoma. *J. Clin. Endocrinol. Metab.* 57:706–712.

21. Clement-Jones, V., P. J. Lowry, L. H. Rees, and G. M. Besser. Development of a specific extracted radioimmunoassay for methionine enkephalin in human plasma and cerebrospinal fluid. *J. Endocrinol.* 86:231–243.

22. Singer, E. A., S. P. Mitra, and R. E. Carraway. 1986. Plasma protein(s) yields Met-enkephalin-related peptides in near-micromolar concentrations when treated with pepsin. *Endocrinology.* 119:1527–1533.

23. Kraemer, W. J., L. E. Armstrong, L. J. Marchitelli, R. W. Hubbard, and N. Leva. 1987. Plasma opioid peptide responses during heat acclimation in humans. *Peptides.* 8:715–719.

24. Lewis, R. V., A. S. Stern, D. L. Kilpatrick, L. D. Gerber, J. Rossier, S. Stein, and S. Udenfriend. 1981. Marked increases in large enkephalin-containing polypeptides in the rat adrenal gland following denervation. *J. Neurosci.* 1:80–82.

25. Steiner, D. S., P. S. Quinn, S. J. Chan, J. March, and H. S. Tager. 1980. Processing mechanisms in the biosynthesis of proteins. *Ann. N.Y. Acad. Sci.* 343:1–16.

26. Carraway, R. E., S. P. Mitra, and D. E. Cochrane. 1987. Structure of a biologically active neurotensin-related peptide obtained from pepsin-treated albumin(s). *J. Biol. Chem.* 262:5968–5973.

27. Rocha e Silva, M., W. T. Beraldo, and G. Rosenfeld. 1949. Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin. *Am. J. Physiol.* 156:261–273.

28. Gross, F., and H. G. Vogel. 1980. Enzymatic Release of Vasoactive Peptides. Raven Press, New York.

29. Mousa, S. A., and G. R. Van Loon. 1985. Acid-lyophilizable Met-enkephalin in plasma: an index of total plasma Met-enkephalin. International Narcotics Research Conference, North Falmouth. 193. (Abstr.)

30. Giraud, A. S., L. Parker, C. Reichman, M. Familari, A. I. Smith, and J. Funder. 1989. Generation of Met-enkephalin Arg^6Phe^7 immunoreactivity by proteolytic cleavage of mammalian plasma precursors by pepsin. *Endocrinology.* 124:1711–1716.

31. Pierzchala, K., A. Houdi, and G. R. Van Loon. 1987. Nicotine-induced alterations in brain regional concentrations of native and cryptic Met- and Leu-enkephalin. *Peptides.* 8:1035–1043.

32. Boarder, M. R., E. Erdelyi, and J. D. Barchas. 1982. Opioid peptides in human plasma: evidence for multiple forms. *J. Clin. Endocrinol. Metab.* 54:715–720.

33. Shen, F.-S., and I. Lindberg. 1988. Characterization of enkephalin-immunoreactive peptides generated by peptic digestion of rat plasma proteins. *Endocrinology.* 122:2905–2910.

34. Possenti, R., V. DeMarco, O. Cherubini, and L. G. Roda. 1983. Enkephalin-binding system in human plasma. *Neurochem. Res.* 8:423–432.

35. Nielsen, A. H., C. Malling, and K. Poulsen. 1978. Characteristics and conversion of high molecular weight forms of renin in plasma and their incomplete activation by the current acid treatment. *Biochim. Biophys. Acta.* 534:246–257.

36. Poulsen, K., J. Kroll, A. H. Nielsen, J. Jensenius, and C. Malling. 1979. Renin binding proteins in plasma: binding of renin to some of the plasma protease inhibitors, to lipoproteins, and to a non–trypsin-binding unidentified plasma protein. *Biochim. Biophys. Acta.* 577:1–10.

37. Van Loon, G. R., K. Pierzchala, L. V. Brown, and D. R. Brown. 1988. Plasma Met-enkephalin and cardiovascular responses to stress. *In* Opioid Peptides and Blood Pressure Control. K. O. Stumpe, K. Kraft, and A. I. Faden, editors. Springer-Verlag, Berlin. 117–126.

38. Owens, P. C., E.-C. Chan, M. Lovelock, J. Falconer, and R. Smith. 1988. Immunoreactive Methionine-enkephalin in cerebrospinal fluid and blood plasma during acute stress in conscious sheep. *Endocrinology.* 122:311–318.

39. Medbak, S., D. F. J. Mason, and L. H. Rees. 1987. Plasma Met-enkephalin and catecholamine responses to insulin-induced hypoglycemia in greyhounds. *J. Endocrinol.* 114:81.

40. Engeland, W. C., D. P. Dempsher, and D. S. Gann. 1981. The adrenal medullary response to graded hemorrhage. *Endocrinology.* 109:1539–1544.

41. Grodsky, G. M., H. Sando, S. Levin, J. Gerich, and J. Karam. 1974. Synthesis and secretion of insulin in dynamic perfusion systems. *Adv. Metab. Dis.* 7:155–168.

42. Van Loon, G. R., K. Pierzchala, P. Zeman, and R. Kvetnansky. 1989. Plasma native and peptidase-hydrolyzable Met-enkephalin: adaptation of the responses to restraint and immobilization stress in rats. *In* Stress, Neurochemical and Humoral Mechanisms. G. R. Van Loon, R. Kvetnansky, R. McCarty, and J. Axelrod, editors. Gordon and Breach Science Publishers, Inc., New York. 670–689.

43. Van Loon, G. R., J. Kiritsy-Roy, K. Pierzchala, L. Dong, F. A. Bobbitt, L. Marson, and L. Brown. 1989. Differential brain and peripheral nicotinic regulation of sympathoadrenal secretion. *Prog. Brain Res.* 79:217–223.

44. Hexum, T. D., H.-Y. T. Yang, and E. Costa. 1980. Biochemical characterization of enkephalin-like immunoreactive peptides of adrenal glands. *Life Sci.* 27:1211–1216.

45. Barron, B. A., K. Pierzchala, and G. R. Van Loon. 1987. Sympathetic nerves, not adrenal medulla, are the source of the stress-induced increase in plasma Met-enkephalin. *Progr. 69th Ann. Meet. Endocrine Soc.* 206. (Abstr.)

46. McCarty, R., and E. A. Stone. 1984. Chronic stress and regulation of the sympathetic nervous system. *In* Stress. The Role of Catecholamines and Other Neurotransmitters. E. Usdin, R. Kvetnansky, and J. Axelrod, editors. Gordon and Breach Science Publishers, Inc., New York. 563–576.

47. Holaday, J. W. 1983. Cardiovascular effects of endogenous opiate system. *Annu. Rev. Pharmacol. Toxicol.* 23:541–594.

48. Van Loon, G. R. 1984. Endogenous opioid peptides in the regulation of arterial blood pressure. *In* Hypertension and the Brain. G. P. Guthrie, Jr., and T. A. Kotchen, editors. Futura Publishing Co., Inc., Mt. Kisco, New York. 127–154.

49. Weigant, D. A., and J. E. Blalock. 1987. Interactions between the neuroendocrine and immune systems: common hormones and receptors. *Immunol. Rev.* 100:79–108.